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PROGRESS IN THE STANDARDIZATION OF STAINS

A NEW LOCATION FOR THE CHEMICAL WORK

A year ago[1] announcement was made in these pages of the end of the coöperation with the U.S. Department of Agriculture, through whose agency the chemical and spectrophotometric work of the Stain Commission had been carried on in the past. Immdiately after this relation came to an end, the chemical work was put temporarily in the hands of a firm of analytical chemists, while the spectrophotometric analyses were transferred to Geneva.

Gradually the work carried on at Geneva has been increased in scope until it has come to include not only the chemical assay of samples submitted but a broad field of chemical and spectrophotometric research. This work has been under the supervision of Dr. E. H. Stotz, who is the representative on the Commission of the American Chemical Society; and his interest in the field investigated has proved of much value to the Commission.

Now it is necessary to move the work again; but fortunately in so doing the assistance of Dr. Stotz will be retained. He has just left the New York State Agricultural Experiment Station to accept a position as head of the Department of Biochemistry at University of Rochester Medical School. He is taking with him the chemical and spectrophotometric work of the Commission, primarily as a research program to be undertaken in the Department of which he is to be in charge; but it will be possible for him to include with the research work, the routine assay of such samples as are submitted to the Commission for testing. This work is to be financed by means of a grant made by the Stain Commission to the University of Rochester.

It is felt that this will be a very desirable arrangement from the standpoint of the Commission. It was understood from the beginning that the location of the chemical work at Geneva was only temporary. Nevertheless, although this arrangement has continued less than two years, it has enabled us to get the chemical and biologi-

[1]Conn, H. J. 1946. Twenty-five years of cooperation with the U. S. Department of Agriculture. Stain Techn., 21, 81–6.

cal work knit together in a closer fashion than ever before, and there is no reason why the two lines of investigation cannot continue in the future in as close harmony as has been the case this last year.

As a matter of fact, some of the biological work will now be carried on at Rochester, also; for Miss Mary A. Darrow, who has been a valued employee of the Commission for some time, is also moving to Rochester and will continue her work for us there. This latter change might cause some inconvenience but for the fact that Rochester and Geneva are so close together that the work will remain in almost as close touch with the Commission headquarters at the latter place as it has been in the past.

Under this new arrangement, it is planned to carry on at Rochester a considerable program of research, largely concerning reasons for variations in the chemistry and optical properties of various samples of any dye, and the relation of such variations to their staining properties. Coöperation of other departments of the Medical School at Rochester has been promised; and it is hoped that the research can, therefore, cover a broader field than would be possible if it were continued at Geneva.

Grants for Research Work

Elsewhere in these pages last year (p. 42, April, 1947) a brief note was made of a grant to Northwestern University Medical School for investigations in neurological microtechnic under the supervision of Dr. H. A. Davenport. This is proving a prolific bit of research; two papers have already been published, one in the April number of this journal, the other in the current issue.

The papers already published have dealt with the stainability of nerve fibers by protargol and by methylene blue, respectively; both of these fields of investigation are being continued. In addition to this program, Dr. Davenport reports his intention of investigating the Feulgen reaction as a possible block staining method for general histological purposes. The grant made for this work in 1946 has been renewed this year, thus extending the program into 1948.

Another such grant, in a different field, is now under consideration.
—H. J. Conn.

DIFFERENTIAL CYTOPHYSIOLOGICAL DIAGNOSIS OF CANCEROUS AND NORMAL TISSUES

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Abstract.—**A method is offered for the differential diagnosis of cancer cells. It depends on the use of methylene blue decolorized with sodium thiosulfate (denoted here HLM, i.e. "hyposulfite methylene blue"); this is prepared by dissolving 800 mg. sodium thiosulfate in 10 ml. of 0.1% aqueous methylene blue and adding 3-5 drops of dilute (1:3) HCl. Frozen sections are treated with this reagent for 2-3 minutes, rinsed with a large amount of distilled water, then stained 2-3 minutes with 0.05% aqueous acid fuchsin. Staining should be performed in a darkened room. If all due precautions are observed, normal tissue appears blue, malignant tissue red.**

According to Unna (1916), the nuclei of most diverse normal tissues are stained intensely blue with the leucobase of methylene blue. By means of this leucobase (Rongalitweiss) a peculiar phenomenon was found, viz. in normal cells the cytoplasm stains light blue and the nuclei dark blue, while in the cells of malignant tissues, the cytoplasm either remains colorless or assumes a light bluish tint, and the nucleus as a rule remains colorless (Roskin, 1931, 1932, 1937; Roskin and Solowjewa, 1935). This phenomenon was confirmed by Waterman (1934) and Voinov (1940). It seemed the more interesting as the positive reaction with Rongalitweiss depends on the presence, in the normal cells, of an enzyme (an oxidoreductase). That is to say, we are dealing here not merely with microtechnical staining of the common type, but with a peculiar cytophysiological staining reaction of normal and malignant cells.

This conclusion is further supported by the fact that the staining reaction of normal cells with Rongalitweiss is prevented by heating to 80° C. for 30 minutes, as well as by alcohol, formol or treatment with KCN ($M/1000$) for 40 minutes or by a 30 minute exposure to ultraviolet. Of course, it is easy to show that none of these factors exert any apparent effect upon the common staining of the cells with methylene blue. Thus, a negative reaction of malignant cells to Rongalitweiss not only indicates its cytophysiological peculiarity but may also be used for differential diagnosis.

It is worth mentioning that the nuclei of many Protista, unlike those of the Metazoa, show a staining reaction similar to that of malignant cells. This fact may prove of interest for further study (Roskin and Semenoff, 1933).

The application in this way of Unna's Rongalitweiss for cytodiagnostic purposes may, however, involve certain difficulties. A new

technic has therefore been developed in which the hyposulfite (i.e. sodium thiosulfate) leucobase of methylene blue (HLM) is used as the basic reagent. We believe that the new method secures rapid differential staining of normal and malignant cells and tissues. The HLM method may prove more than an aid in the routine histopathological practice, especially for diagnosis of malignant cells in exudates and punctates. Indeed, it may become a useful tool for studying the properties of cells in diverse forms of malignant and benign tumors, in precancerous states, etc. The value of this method of cytodiagnosis of malignant tumors has been checked on extensive material of the Moscow Oncological Institute, and Dr. Voinov (Thesis for a Doctor's degree, 1941, personal communication) has obtained very useful results in over 300 cases of diverse malignant and benign tumors.

Preparation of the Hyposulfite Leucobase of Methylene Blue (HLM)

To prepare the leucobase of methylene blue, the following mixture is used: 800 mg. sodium thiosulfate, ($Na_2S_2O_3 \cdot 5H_2O$) 10 ml. of 0.1% aqueous methylene blue and 3–5 drops of hydrochloric acid (sp. gr. 1.17) diluted 1:3 with distilled water. The staining results are best with 4 drops of HCl; with less acid the leucobase becomes unstable and is soon transformed into a light blue solution which is no longer good for staining. The methylene blue solution is prepared with redistilled water and is thoroughly filtered before use.

Sodium thiosulfate should be chemically pure (C. P. grade); the commercial "hypo" should be dissolved in distilled water, filtered and recrystallized, the dry crystals being kept in a tightly stoppered jar.

In preparing the leucobase the following directions should be strictly observed: 800 mg. sodium thiosulfate are put in a clean flask (this should be absolutely clean and dry) to which 10 ml. 0.1% aqueous methylene blue are added. As soon as the thiosulfate is completely dissolved (shake several times) 4 drops of diluted HCl are added to the flask. The solution is agitated while being carefully heated over a burner (20–30 seconds) until the appearance of a gray-milky sediment. The solution is then cooled (5–10 minutes) and thoroughly filtered through a double filter. The funnel, like the vessels generally, should be absolutely clean with no traces of methylene blue; otherwise the filtered leucobase would become blue. If the filtered leucobase assumes a blue tint, it should stand for a while in the dark. The methylene blue leucobase should be prepared not long before use but it can be kept in the dark for 10–12 hours. The properly prepared leucobase is colorless or slightly yellowish. Only such a leucobase is suitable for staining sections.

Preparation of Sections

The sections are prepared by means of a freezing microtome from fresh non-fixed tissue fragments. The thickness of the sections is 10–15 μ. Pieces of normal or pathological tissues may be kept before use in a refrigerator (–2 to +2 C°.) in physiological saline for a period of 1–2 days.

The sections are transferred to physiological saline (in a Petri dish) and from there, by means of a pipette, to a slide (excess of physiological saline is sucked off with filter paper). They are then spread over the slide by means of needles and dried at room temperature. Beside the section of a tumor or other pathological tissue, a section of normal tissue is attached to the slide (e.g. of kidney or heart of a mouse) and serves as control of the staining effect. Such dried sections are ready for staining, either immediately or one or two days thereafter. The dried sections of malignant tumors preserve their typical staining capacity for two weeks provided they are kept in a dry and dark place.

Staining of Sections

The leucobase solution is pipetted onto a slide with sections attached. This leucobase should remain colorless throughout staining. If the leucobase solution becomes bluish, it should be changed. Staining should be continued until the section of the normal tissue stains a distinct blue while the section of the malignant tissue remains colorless—usually not more than 2–3 minutes. The leucobase is then poured off, the sections rinsed with a large amount of redistilled water after which the section is treated with 0.05% aqueous acid fuchsin (fuchsin S) for 2–3 minutes. Overstaining should be avoided. The acid fuchsin is then poured off and the sections well dried. The sections may be examined in glycerol or embedded in glycerol-gelatin. Permanent preparations may also be made by embedding the dried sections in pure paraffin oil and sealing the cover slip with a proper medium (asphalt varnish, etc.). The sections may also be embedded in Canada balsam but this should be neutral; otherwise the sections of cancerous tissues soon become blue.

Staining with the leucobase of methylene blue requires strict illumination conditions; the whole staining process should be carried out in a darkened room. Bright illumination may distort the staining effect as the leucobase solution is changed by light. Finally, we repeat: to avoid erroneus results of staining, the sections should be stained only with a colorless solution of the leucobase. With the proper observance of the above directions the difference between

malignant and normal tissue can also be observed macroscopically, viz. normal tissue appears blue and malignant tissue red.

The above HLM method is applicable not only to sections, but also to smears of various normal and pathological tissues.

Results of Staining

The *nuclei of normal cells stain dark blue while the nuclear structures become quite distinct;* the nuclei of cancerous cells either remain *almost colorless* or assume a diffuse *hardly noticeable light blue* or a slightly perceptible pale pink color. The nucleoli of malignant cells appear more intensely colored than the almost colorless nuclei although their blue color is far below that of normal nucleoli. The protoplasm of normal and malignant cells is stained distinctly red by fuchsin S.

The conclusion is thus indicated that *in contrast to the nuclei of normal cells those of malignant cells are unable to transform the colorless hyposulfite leucobase of methylene blue into the colored form.*

A combination of this method with that of MacCarty (increased size of nucleoli of cancer cells) may presumably yield rapid and relatively reliable results. See MacCarty (1928, 1929), MacCarty *et al.* (1933, 1934).

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SOME SYNTHETIC RESINS IN COMBINED FIXING, STAINING AND MOUNTING MEDIA

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ABSTRACT.—**Many of the recently devised plasticizers and resins can be utilized to advantage in cytological technics. Some of them have solubilities which enable us to incorporate them in such fixing and staining solutions as aceto-carmine and propionic-carmine. They are non-volatile, do not alter the fixation images of the fluids with which they are mixed, and serve as mounting media as the volatile components evaporate. Thus it is possible to make a permanent slide in a single operation. These newer compounds are better adapted for this technic than are the natural balsams which have been used previously, as their greater tolerance for water provides a much greater margin of safety. Procedures are described for the utilization of (1) Rezyl 7020, a water-soluble resin (now, unfortunately, not available), which dries to form a water insoluble film, (2) Amberol 750 and (3) Bakelite BR–7160, two alcohol soluble resins, more miscible in solutions containing water than are the natural balsams. Formaldehyde can be included in the aceto-carmine and propionic-carmine fluids with the result that more nuclear detail is preserved. Lacto-gelatin has some valuable properties as a mounting medium and can be used when the specimen is stained with orcein. Carmine, which gives a permanent stain in Rezyl 7020, Amberol 750 and Bakelite BR–7160 fades in lacto-gelatin.**

Several years ago (1937, 1940) the writer described a number of mixtures which could fix and stain chromosomes and also serve as mounting media when the volatile components evaporated. The object of devising such fluids was to make possible the transforming of smear preparations into permanent mounts with the least possible effort. The technic as described has had a limited success, but the margin of safety was small in every case, and the careful work which was often required to make a permanent slide in a single operation defeated the chief objective of the method.

The mounting media tested were of two types, water-soluble and fat-soluble. The former included dextrin, dextrose, gelatin, gum arabic, pectin, sorbitol and sucrose. The fat-soluble mounting media were Canada balsam, sandarac, and Venetian turpentine. The water-

soluble media, with the exception of gum arabic, could be incorporated in the usual aceto-carmine fixing fluids. The uses and limitations of these liquids have already been listed (Zirkle, 1937,1940). It is sufficient to record here that it is difficult to add enough of the nonvolatile component to an aceto-carmine fixative to serve as an adequate mounting medium without altering the fixation image and preventing the swelling of cells and chromosomes which is such a valubable aspect of acetic acid fixation. Several investigators (Sax, 1937, La Cour, 1941, 1947, Speese, 1945) have corrected this fault by fixing with aceto-carmine or aceto-orcein and using one or another of the mixtures simply as a mounting medium.

The method of dissolving Canada balsam, etc., in fixing and staining solutions which contain water has been described in detail by the writer (1940) and need not be repeated. The limiting factor here is the amount of balsam and water which these fluids can hold together in solution. As the mixtures must also give a satisfactory fixation image and dissolve enough of the dye to stain the chromatin, they have to perform a number of functions simultaneously. As every component must be in sufficient quantity to function adequately, yet not in a concentration which will interfere with the reactions of other reagents, there is very little leeway possible in the proportions of the several ingredients. Wilson (1945) has avoided the worst limitations of the method by fixing, staining and mounting in different steps and thus he has extended the utility of the procedure.

During the past two years many new plastics, plasticizers and synthetic resins have become available. It was thought that a number of these might have the solubilities which would allow them to be incorporated in fixing, staining and mounting media and that such mixtures could be used with a greater margin of safety than any which have previously been described. The idea was fully justified; in fact, nearly all the plasticizers tested could be added in sufficient quantity to aceto-carmine without altering the fixation image and thus durable liquid mounts could be made which would last, with normal precautions, for several weeks. Some of the plasticizers could be combined with gum kauri, a natural resin, and permanent mounts could be made requiring fewer precautions than are necessary when Canada balsam, sandarac or Venetian turpentine were employed. There is no need to record these procedures in detail, however, for several of the synthetic resins proved to be so satisfactory that there seems to be little need for the liquid mounts.

It should be emphasized that the primary object of a single fluid technic is that it be quick and easy and that it require no special

precautions. Of course the fixing, staining and the final mounts should be at least as satisfactory as those obtained by other methods, but the introduction of several steps into the process to make it fit difficult biological material sacrifices one of its chief advantages. Small changes in the composition of the fluid itself should be sufficient to extend its applicability to different types of cells.

The proportions of the several components in the following mixtures were derived empirically, the pollen mother cells of *Tradescantia* being used as test material. For other material it is possible that these proportions could be altered to advantage.

Only three of the synthetic resins which were investigated are described here. Each has its particular advantages and disadvantages. The introduction of orcein as a cytological stain by La Cour (1941) has made it possible to mount stained specimens which keep their color in a lactic-acid gelatin mixture in which carmine fades. As this medium has its own particular virtues, its description is included.

Rezyl 7020[1]

Rezyl 7020 is a water-soluble alkyd resin which has the very special property of drying into a water-insoluble film. Its index of refraction is approximately 1.484 but may vary slightly from batch to batch. After completing the work with this product, however, it was discovered that Rezyl 7020 is no longer on the market. A letter from the American Cyanamid and Chemical Company states that its manufacture has been discontinued, at least temporarily, due to two factors: (1) one of the necessary ingredients could no longer be obtained and (2) no large scale demand for the products developed. There seems to be little prospect for its manufacture being resumed in the immediate future although it may become available later. The following abbreviated account of the Rezyl technic is printed with the hope that it may be useful either if Rezyl 7020 again becomes available or some other resin is found with like properties.

Rezyl 7020 is soluble and stable in mixtures of acetic acid and water, and can be added directly to aceto-carmine. Three parts of aceto-carmine to one of Rezyl makes a workable mixture but a sharper fixation image is secured by increasing slightly the amount of acid. As formaldehyde and acetic acid together preserve more than either reagent does when used alone, the incorporation of

[1]A product of the American Cyanamid and Chemical Corporation, 30 Rockefeller Plaza, New York 20, New York. The writer is indebted to Mr. J. M. Cochran of the Organic Chemicals Dept. for supplying this resin.

formaldehyde with acetic acid in the usual smearing technic results in the preservation of more nuclear detail. Mixtures of acetic acid and formalin (40% aqueous solution of formaldehyde) in various proportions will dissolve sufficient carmine so the fixing and staining may be accomplished simultaneously. The following mixture is recommended:

Rezyl 7020	25 ml.
Glacial acetic acid	30 "
40% formaldehyde	70 "
$Fe(NO_3)_3 \cdot 9\ H_2O$	0.3 g.
Carmine	To saturation

Specimens fixed in the above are firmer and more resistant to distortion than they are when formaldehyde is omitted. Of course the Rezyl need not be included and the fluid can then be used to advantage for temporary smears.

Orcein can be used in place of the carmine. Flaming improves the quality of the fixation and secures the characteristic swelling of the aceto-carmine method. Cells can be flattened by applying pressure to the cover glass but when this is done an extra drop or two of the fluid should be placed at the edges of the cover glass to replace the liquid lost before the preparation dries.

Advantages: The chief advantage of the Rezyl technic is its simplicity and the ease with which it can be incorporated into a familiar procedure. As moisture does not hurt the mount no precautions need be taken for drying even in the extremely humid air of seaside laboratories. Once the Rezyl has hardened the mount is almost as impervious to water as some of the fat-soluble media. The mounting medium itself may constitute as much as 25% of these mixtures, as compared with 5–16% of the other water-soluble media. This gives sufficient leeway for decreasing the Rezyl in fluids designed for very delicate material.

Disadvantages: The chief disadvantage in the use of Rezyl lies in the relative slowness with which it solidifies under a cover glass. In addition it is not very tacky and does not adhere to glass as strongly as balsam but if the smear is thin and enough of the medium has been used, no air need be drawn under the cover glass as the preparation dries. When solid, Rezyl is not as hard as balsam but it is firm enough for ordinary microscopic preparations. When formaldehyde is included in the mixture, the cytoplasm stains as well as the chromatin. The stain, however, is lighter and need not cause trouble.

Amberol 750[2]

Amberol 750 is a clear, transparent, alcohol-soluble resin with an index of refraction of 1.536 and a relatively high melting point. It is insoluble in water and in aromatic and aliphatic hydrocarbons but it is soluble in mixtures of phenol, propionic and acetic acids and water. It is not soluble in the usual aceto-carmine and propionic-carmine fluids but very soluble in those which dissolve Venetian turpentine. It is a light straw color in solution and dries to films which are practically colorless.

The principles involved in incorporating a fat-soluble resin in a fixing and staining mixture have been discussed in some detail (Zirkle, 1940). The formulas for mixtures containing Amberol 750 are essentially like those for Canada balsam, sandarac or Venetian turpentine except that minor changes can be made to take advantage of the much greater tolerance of Amberol 750 for water in the solution. The following proportions are suggested as a starting-point but it is emphasized that these proportions may be altered to advantage to fit particular research problems.

Amberol 750	20 g.
Phenol (crystals)	35 ml.
Propionic acid	20 ml.
Acetic acid	5 ml.
Water	20 ml.
$Fe(NO_3)_3 \cdot 9\ H_2O$	0.4 g.
Carmine	To saturation

The above mixture is satisfactory under a variety of conditions. A still greater tolerance for water can be secured, however, if some relatively non-volatile liquid such as "Carbitol" (diethylene glycol monoethyl ether) or dimethoxytetraethyleneglycol[3] be substituted for a part of the Amberol. Thus 15 grams of Amberol plus 5 ml. of Carbitol will dry to form a solid, firm and clear mounting medium even if the mixture contains as much as 25 ml. of water. The extra water may be necessary if the specimen to be fixed is extremely delicate.

Orcein should not be substituted for carmine in this formula. It offers no advantage as the carmine practically never stains the cytoplasm but only the chromatin. Then too, if carmine is used, the resin is practically colorless when it hardens, but with orcein it re-

[2]Amberol is made by the Resinous Products and Chemical Co., Washington Square. Philadelphia, Penna. The writer wishes to thank Mr. Philip B. Taft of this company for samples of Amberol.

[3]These compounds can be purchased from the Carbide and Carbon Chemicals Corporation. 30 E. 42nd St., New York 17, N. Y.

mains heavily stained for some time. The Amberol will go into solution quicker if it is ground in a mortar before it is added to the mixture. It should dissolve completely in 12 hours. The iron mordant should be dissolved before the carmine is included. More carmine should be added than will dissolve. The fluid need not be filtered as the excess carmine will settle to the bottom of the container and the liquid will clear. This fixative should be employed in the same volume as the Rezyl solutions. Flaming the slide is neither necessary nor desirable.

Advantages: As a mounting medium Amberol 750 has the advantages of the natural balsams. The combined fixing, staining and mounting media which have been described previously could not contain safely more than from 13–14% of the natural balsams. If more were added the preparation would frequently become opaque in drying, but mixtures can be made which contain more than 25% Amberol, and dry to perfectly transparent films. In moist atmospheres, slides made from the balsam mixtures had to be dried on hot plates or even in paraffin ovens, a procedure which is not necessary for the Amberol mounting. No mounts in Amberol have clouded even when they were exposed to moist summer weather while they were drying. When dry, Amberol is hard and adheres firmly to glass. The fixing mixture which dissolves Amberol has many advantages over 45% acetic acid as it preserves the chromatin with a sharper outline and shows more detail.

Disadvantages: The chief limitation to the use of Amberol is inherent in the incorporation of a water-insoluble substance in a fluid containing water. It should be stated, however, that the test slides have been made carelessly, with no particular precautions and they have been exposed to rough usage but they have not deteriorated. Possibly under humid tropical conditions the mounts would have to be dried artificially. Sometimes whole uncrushed cells in slides which have been aged by exposure to a large daily fluctuation in temperature lose some of the original swelling but chromosomes thus mounted have kept their characteristic structure.

Bakelite BR–7160[4]

Bakelite BR–7160 is an opalescent, alcohol and fat soluble resin with an index of refraction of 1.652. When dissolved in fixing and staining fluids it makes them cloudy, and seemingly unfit for micro-

[4]A product of Bakelite Corporation, 30 E. 42nd St., New York 17, New York. The writer is indebted to Mr. Preston H. Scott and Dr. H. L. Bender for their courtesy and assistance and for supplying this resin.

scopic preparations. The solution, however, can be clarified by filtering and the filtrate remains completely transparent even when dried and hardened. Bakelite BR–7160 is more tolerant of water than Amberol 750. On the other hand, comparable solutions of Bakelite dissolve less carmine so it is necessary, if the Bakelite preparations are to be stained sufficiently, to alter the proportions of the other ingredients so that more carmine will dissolve. This can be done by increasing the relative amount of water. The following preparations are recommended.

Bakelite BR–7160	20 g.
Carbitol	5 ml.
Phenol	30 ml.
Propionic Acid	20 ml.
Acetic Acid	5 ml.
Water	20 ml.
$Fe(NO_3)_3 \cdot 9\ H_2O$	0.4 g.
Carmine	To saturation

The phenol, acids and water should be mixed first and then the ground up Bakelite and the iron salt added. It is best to agitate the fluid so that the Bakelite does not form large clumps. After the iron has dissolved, the carmine should be added. The solution should not be filtered until all of the Bakelite has dissolved and until it has become saturated with carmine. Twenty-four hours is generally long enough to wait before filtering. The fluid should not be heated.

Bakelite is not soluble in aceto-carmine but is soluble in propionic-carmine. The writer has pointed out (1933,1940) the possibility of substituting propionic for acetic acid in the usual cytological fixatives and also some of the advantages of the substitution. Forty-five per cent acetic acid (by volume), the standard amount in aceto-carmine, is approximately 8 molar. An 8 molar concentration of propionic acid would be, in round numbers, 60% (by volume). This strength propionic acid will dissolve enough Bakelite to serve as a practical mounting medium. If a 40% solution of formaldehyde be used in place of the water, the fluid gives a fixation image which shows more nuclear detail. The following proportions have proven satisfactory.

Bakelite BR–7160	25 g.
Carbitol	10 ml.
Propionic acid	60 ml.
40% Formaldehyde	40 ml.
$Fe(NO_3)_3 \cdot 9\ H_2O$	0.4 g.
Carmine	To saturation

This Bakelite solution can not absorb as much water as the one which contains phenol. However, if the specimen is not large and elementary precautions are observed not to bring extra water on to the slide with the specimen, there should be no trouble. No special precautions are necessary in drying the slides. If any air is drawn under the cover glass as the preparation ages, more Carbitol should be added to the fixing mixture.

Advantages: Bakelite has essentially the same advantages as Amberol. In addition its greater tolerance of water allows it to be dissolved in 60% propionic acid and thus it can be incorporated in a propionic-carmine solution, and in a fixing and staining mixture which contains formaldehyde.

Disadvantages: Bakelite likewise has the limitations of Amberol. Some of the margin of safety gained by its use is lost through the necessity of increasing the proportion of water in the mixture so that enough carmine will dissolve to stain the specimen adequately. The Bakelite fluids have to be filtered which is not necessary with those which contain Amberol. It is also necessary that a non-volatile liquid such as carbitol be incorporated to prevent the mount from cracking with age. Carbitol is also required in the propionic acid formaldehyde mixture to keep the preparation from clouding as it hardens. While formaldehyde increases the amount of nuclear detail preserved, it fixes the cytoplasm so that it stains with carmine. Orcein, in the Bakelite mixtures gave very erratic results. Films of Bakelite dried on glass and exposed to light and air acquire a brownish tint. This discoloration has not yet been observed to occur under cover glasses but there is a definite possibility that microscopic preparations of Bakelite will be discolored in time. Thus far, the discoloration does not appear to be sufficient to interfere in any way with the visibility of the mounts.

Lacto-Gelatin

Gelatin has long been a component of mounting media. When used by itself it becomes too hard and brittle for permanent preparations but when mixed with glycerin and boiled in 45% acetic acid it acquires a satisfactory consistency. The chemical changes induced by the boiling in acid need not concern us here. Unfortunately glycerin and the other non-volatile liquids which give the mounts a proper consistency plasmolyze any cells not broken by the smearing technic unless they are incorporated in the fixing and staining fluids in small mounts. Thus the media cannot contain much of the non-volatile components, 8% of the total volume being the upper usable

limit for glycerin while the glycerin and gelatin together should not exceed 15%. This means that the final volume of the preparation will be less than one sixth of that of the fluid in which the specimen is mounted. Unless the smear is clean and thin, air may be drawn in under the cover slip as the preparation dries. The necessity of any extra precaution limits the applicability of the technic.

Lactic acid is a non-volatile liquid which forms an excellent mounting medium with gelatin — firm, tacky and lasting. Mixed with acetic acid it apparently penetrates more rapidly than glycerin and can be used in much greater concentrations without causing the cells to shrink. Its use was not advised, however, (Zirkle, 1940) for it destroyed the color of aceto-carmine preparations; no matter how well the chromosomes were stained originally, they were colorless after twelve hours in lacto-gelatin.

The introduction of aceto-orcein by La Cour has given this mounting medium a certain value. La Cour has reported that orcein — dissolved in equal parts of glacial acetic acid, lactic acid and water—will fix and stain chromatin, and that such preparations will last for a week or two. The incorporation of gelatin into the mixture will make such mounts permanent. Thus 10 grams of gelatin added to 30 ml. each of acetic acid, lactic acid and water form a usable medium, which, however, contains more lactic acid than is needed. The following proportions are less apt to cause unbroken cells to shrink.

Gelatin	10 g.
Lactic acid	15 ml.
Acetic acid	30 ml.
Water	45 ml.
Orcein[5]	To saturation

The gelatin should first be dissolved in the water and the acids and orcein added. The whole should be boiled for two or three minutes. It is not necessary to filter as the undissolved orcein will settle to the bottom of the container. For exceptionally delicate material the mixture can be diluted with ordinary aceto-orcein. Flaming is a necessary part of the fixation.

Advantages: Lacto-gelatin is firmer and more tacky than Rezyl 7020 and hardens much faster. As it is soluble in water it can be used under almost any conditions.

Disadvantages: As stated, lacto-gelatin cannot be used with carmine. When the mounts are exposed daily to direct sunlight even the orcein fades in about two months. Slides kept away from the

[5]The writer employed a Coleman and Bell orcein which is understood to be a recently imported product (non-synthetic).

light have preserved their color, although it is too soon to state whether or not the stain is permanent.

The following medium is suggested as a temporary substitute for Rezyl 7020. As it contains formaldehyde the amount of gelatin must be reduced for, even in the presence of the acids, the formaldehyde will cause the gelatin to solidify if more than the indicated quantity is used.

Gelatin	5 g.
Lactic Acid	30 ml.
Acetic Acid	30 ml.
40% Formaldehyde	70 ml.
Orcein	1 g.

The gelatin and orcein should be dissolved in the acids. Heating the mixture is advised. The formaldehyde should not be added until it has cooled.

Advantages: This mixture gives the fixation image of acetic acid formaldehyde.

Disadvantages: The medium is not as firm as the lacto-gelatin preparation which contains more gelatin and less lactic acid. If the amount of lactic acid is reduced crystals will form as the mount hardens. The staining has thus far proven to be erratic.

General Considerations

The advantages of a single fluid which fixes, stains and serves as a mounting medium are obvious. The chief value of such a one-process technic would be lost, however, if it were necessary to employ it with great care or to take extra precautions. Thus the greater its margin of safety the easier and more widespread should be its use. The four mounting media described here are, within their listed limitations, a marked improvement over those described earlier (Zirkle, 1937, 1940).

How permanent the preparations are it is impossible to say. Slides have been exposed for over a year without protection in a south window, where they are subjected to periodic direct sunlight, and have not deteriorated. (Except that the lacto-gelatin-orcein slides have faded.)

The cytoplasm stains with carmine in those preparations which contain formaldehyde but not in those which contain phenol. Both types of preparations show more nuclear detail than is preserved by 45% acetic acid.

Many of the promising newer synthetic resins have not been tested. There are probably many others now available which are just as useful as those described, and newer ones will probably be even more

serviceable. It seems to be only a question of time until some resin is found which will have all the advantages and none of the limitations of those described here.

The writer wishes to thank Dr. John G. Miller for measuring the indices of refraction of Amberol 750 and Bakelite BR–7160.

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ELIMINATION OF DISTORTION AND POOR STAINING IN PARAFFIN SECTIONS OF PLANT TISSUES

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Abstract.—**Three fixing solutions causing least distortion and bright staining of plant tissues are named. Glycerin dehydration causes less distortion than a series of alcohol concentrations; 95% alcohol removes some of the glycerin, sets the protoplasm and improves the staining. Absolute alcohol causes distortion and should be avoided. Pure chloroform, as a paraffin solvent, is followed by brighter staining but more distortion than are the butyl alcohols. A schedule resulting in minimum distortion is given. The results are shown in photomicrographs. Brightest staining follows the use of C. P. iron alum and hematoxylin. The use of a paper cup for very gradual change from one liquid to another and as a labor saver is described.**

After conducting numerous experiments over an extended period of time we have obtained considerable evidence regarding factors responsible for distortion and poor staining in paraffin sections of plant tissues. We are offering our results at this time with the hope that they may be helpful to others working in this field.

The first essential for good results is, of course, a good fixing solution. Of the many solutions described in the literature we find Karpechenko's solution[1] to cause the least distortion in the largest number of plants. If differential staining is not sufficiently bright when this solution is used, Flemming's strong (half strength)[1] or Allen's B17 should be tried. The latter two solutions are especially good for fungi. Fixation should ordinarily be for around 48 hours. Since Allen's B17 is not readily found in the literature we are giving its composition below. It should be made up immediately before use, the ingredients being added in the order named.

Saturated solution of picric acid	75 ml.
Formalin	25 ml.
Acetic acid	10 ml.
Chromic acid	1.5 g.
Urea	2 g.

After washing the tissues 6 to 12 hours in running water we come to the problem of dehydration. Many experiments have convinced us that dehydration by means of glycerin causes distinctly less distortion than the long series of ethyl alcohol concentrations commonly used. The glycerin solution is made as follows: Make a 10% aqueous

[1]See Rawlins, T. E. 1933. Phytopathological and Botanical Research Methods. John Wiley and Sons, Inc., New York, N. Y.

solution of glycerin. Add 1 ml. of a 10% solution of thymol in 95% alcohol to each 100 ml. of the glycerin solution to prevent growth of fungi. Fill an evaporating dish with about 100 ml. of this glycerin solution and leave the tissues in the solution until the water has evaporated. This requires about 13 days if the evaporating dish is kept on top of the paraffin oven. A canopy of stiff cardboard or other material should be suspended about 6 inches above the evaporating dish to decrease the amount of dust falling into the evaporating dish. If the blocks of tissue tend to settle on the sides of the evaporating dish during evaporation, push them to the middle of the dish so that they will remain covered by the concentrating glycerin solution.

After all of the water has evaporated the tissues are run through several changes of 95% ethyl alcohol. This treatment removes some of the glycerin, sets the tissues and improves their staining qualities.

The use of absolute ethyl alcohol should be avoided if one desires minimum distortion of tissues. If distortion is of less importance and best differential staining of nuclear structures is a primary consideration, absolute alcohol should follow the 95% alcohol and the absolute alcohol should be followed by a good fat and paraffin solvent such as pure (anesthetic grade) chloroform. Equal parts of absolute ethyl alcohol and 95% alcohol give a mixture that is miscible with chloroform. It is possible that this alcohol mixture may cause less distortion than absolute alcohol. More experiments are needed before we can recommend this mixture. If minimum distortion of tissues and moderately bright staining are desired, we have found 95% ethyl alcohol followed by gradual infiltration with normal butyl alcohol to give best results.

In going from ethyl alcohol to a paraffin solvent best results are obtained if a gradual change is produced by the diffusion of liquids through an onion skin paper cup. This paper is widely used in typing and is therefore readily available. The diffusion method saves much time ordinarily spent in changing a long series of solutions.

The procedure is as follows: Cut the onion skin paper into squares 4 to 6 inches across. Place the basal end of a test tube in the center of a square of paper and press the paper tightly (without moistening) around the test tube to produce a paper cup. (The creases in the paper are made tighter if the test tube having the lower end covered by paper is forced into a vial which is slightly larger than the test tube and is then rolled around to tighten the creases in the paper.) Remove the paper cup and cut off the upper portion with scissors to produce a cup about an inch deep. Add about 17 ml. of paraffin solvent to a test tube having an inside diameter about equal to that of the vial. Place the paper cup in the top of the test tube and place 1 or 2 ml. of the desired strength of ethyl alcohol in the cup by means of a pipette

or medicine dropper. Push the paper cup down until the liquid levels outside and inside the cup are about the same. Place the blocks of tissue in the cup by means of a wire loop such as is used by bacteriologists. (Bend the wire so that the loop is horizontal when the wire is vertical.) Cork *tightly* with a rubber stopper and allow diffusion to go until the alcohol around the tissues has been replaced by the paraffin solvent. (Do not allow the stopper to touch any liquid.)

A similar paper cup technic is used to obtain gradual infiltration of paraffin into the tissues. However in this case a vial about 2 inches deep is used. Here the tissues are in the bottom of the vial in the paraffin solvent and the melted paraffin is poured into the paper cup.

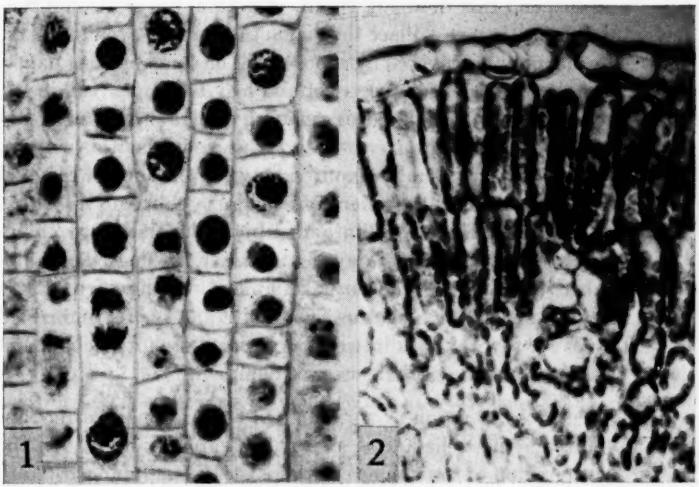

FIG. 1.—Longitudinal section of onion root tip.

FIG. 2.—Cross section of leaf of *Malva parviflora*. (Both sections were prepared by means of the glycerin-95%-alcohol-butyl alcohol combination and were stained with Heidenhain's iron-alum-hematoxylin.)

The paper cup technic should be of wide application in the gradual change from any liquid to another and would save cytologists much unnecessary work in changing long series of solutions.

The following detailed schedule has been more satisfactory for the largest number of plant species than any we have tried:

1. Fix tissues about 48 hours.
2. Wash 6 to 12 hours in running water.
3. Dehydrate the tissues with glycerin.
4. By means of a wire loop transfer tissues to 95% alcohol in a vial. Cork the vial and leave for 2 hours.
5. Give the tissues a second treatment of 1 hour in new 95% alcohol.

6. Place tissues in 1 to 2 ml. of 95% alcohol in an onion skin paper cup suspended in a test tube containing 17 or more ml. of normal butyl alcohol. Stopper the test tube *tightly* and allow diffusion to proceed for 24 hours.
7. Place the test tube on top of the paraffin oven for 2 days to complete the diffusion.
8. Subject the tissues to 2 treatments of new normal butyl alcohol of 24 hours each and 2 treatments of 48 hours each. The vials should be kept on the paraffin oven to hasten the removal of glycerin. Keep the vials corked.
9. Place about 2 ml. of melted paraffin in an onion skin paper cup and suspend this in about 10 ml. of normal butyl alcohol containing the tissues. Place the vial in the paraffin oven for 24 hr. The vial may be covered by a loose cork or small piece of paper.
10. Remove the cork or paper cover and leave the open vial in the oven until the paraffin has reached a constant volume. Remove the paper cup from the vial as soon as it is empty. About 6 days are usually required between the time of removal of the cup and the attainment of constant volume.
11. Pour off the melted paraffin and subject the tissues to 2 one-day treatments and 1 two-day treatment in new melted paraffin.
12. Embed the tissues in the usual manner. (If lignified tissues are too hard to section, the usual softening of the embedded tissues for several days in water or 9 parts of 60% alcohol and 1 part of glycerin should be carried out.)

The absence of distortion or shrinkage of the protoplast after running up through this schedule is shown in figures 1 and 2. In the meristematic region of the onion root there is essentially no shrinkage of the protoplast from the wall. Even in the mature *Malva* leaf the chloroplasts have remained in essentially the same position they occupied in the living cells.

For brightest staining with Heidenhain's iron-alum-hematoxylin the purest grades of iron alum and hematoxylin should be used. We have had best results with "Baker's Analyzed" C. P. ferric ammonium sulfate, $Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$. Only the purple portion of the crystals should be used and the solution should be filtered before using. The hematoxylin should also be of the C.P. grade. The proper sequence of distilled water and tap water[2] is also necessary for best results with this stain.

If one does not desire to wait several months for a hematoxylin solution to ripen, the ripening may be accomplished by drawing air through the solution for 2 or more hours.

[2]See footnote 1.

GALLOCYANIN AS A NUCLEAR STAIN

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Abstract.—**Gallocyanin has been used successfully as a nuclear stain. Sections are cut by the freezing method of either fixed or unfixed tissue. The tissues are warmed (not exceeding 70°C.) for 2–4 minutes in the gallocyanin solution. A counterstain may be used if desired. The most effective are Biebrich scarlet, phloxine, or eosin Y. The sections are then dehydrated and mounted in clarite. The nuclear pattern is clearly demonstrated and the sections are permanent.**

Introduction

Gallocyanin (C.I. No. 883) has been used as a stain by previous workers both singly and in combination. It has been described as a nuclear stain and as a specific stain for the Nissl substance of the motor cells of the central nervous system. Proescher and Arkush (1928) found that the iron lake of gallocyanin was a good nuclear stain and was adequate when substituted for hematoxylin. It has also been used as a constituent of Foley's (1943) combined method for the staining of nerve fibers[1]. Einarson (1932) has used gallocyanin for the demonstration of the Nissl substance. Bruzaglo (1934) used it in combination with orcein and alizarin blue as a tissue stain.

Although hematoxylin is effective in demonstrating the nuclear pattern, it is somewhat difficult to use and does not always give consistent results. It would be advantageous to have a stain for use in normal and pathological histology that would yield consistently a clear nuclear pattern by a simple quick method. In an attempt to supply this need, this paper is concerned with a description of the use of gallocyanin in the staining of frozen sections by the spot plate method of Cole (1947).

Materials and Methods

Stains

It was found that the iron lake of gallocyanin, as described by Proescher and Arkush (1928) washed out of the tissues when used with the spot plate method; even if it were successful the procedure is slow.

[1]Foley (1943) describes alzarin blue in his method for staining nerve fibers. Conn (1946) classifies this as gallocyanin.

Several trials indicated that modification of Einarson's formula (1932) was best for rapid staining. The following formula was used:

Gallocyanin	1.5 g.
Chrome alum	5.0 g.
Distilled water	100 ml.

Mix the chrome alum ($K_2SO_4 \cdot Cr_2SO_4 \cdot 24H_2O$) and the water. Heat to boiling. Add the gallocyanin and boil for about 5 minutes. Filter while hot and store in a dark bottle.

It was found that the stain is rather unstable in solution[2] and should be prepared freshly every two weeks. The dye has a tendency to precipitate out of solution and hence the stain loses its characteristic qualities.

METHODS

Tissues prepared by the frozen section method from 5 to 15 μ in thickness, were put successively in a shallow dish containing 0.9% sodium chloride solution, next in a shallow dish containing 25 ml. of the stain in which they were warmed gently (not more than 70°C.) for 1 to 3 minutes, depending upon the size and type of tissue. Staining can be done in a cold solution but it requires 5–8 hours. The intensity of the stain may be ascertained by removing a section and examining it microscopically. When the staining has progressed sufficiently, the sections are washed in distilled water and dehydrated. Counterstaining is not necessary, if nuclear patterns alone are being studied, but for some tissues it may be desirable to use a counterstain in order to demonstrate the cytoplasm. The sections may be cleared in any of the common clearing solutions. In this laboratory good clearing is obtained by using beechwood cresote (Merck) or this solution:

Xylene	5 parts
Toluene	1 part
Beechwood cresote	1 part
Aniline oil	1 part

In general the following procedures have given the most satisfactory results. The alcohol used was methyl alcohol.

1. Gallocyanin as described.
2. Distilled water, 2 minutes before Biebrich scarlet, (C.I. No. 280) or 15 seconds before phloxine (C.I. No. 778, certification No. NPh 7)
3. Alcohol, 25%; 15 seconds
4. Alcohol, 50%; 15 seconds

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[2]This subject is under investigation and it is hoped to alter the stain so as to increase its stability without losing any of its advantages.

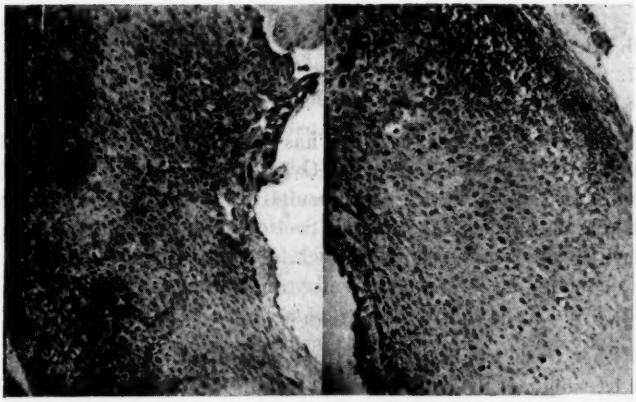

FIG. 1.

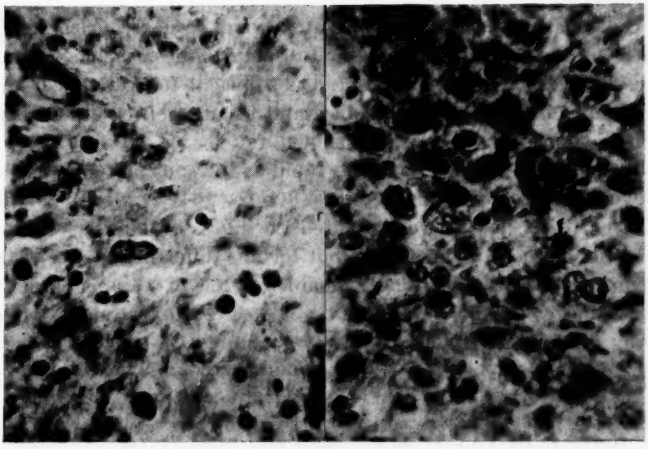

FIG. 2.

Biospy of neoplasm removed from the distal third of the finger over the base of the nail. Photomicrographs demonstrate nuclear pattern stained by gallocyanin with no counterstain. Frozen sections prepared from tissue fixed in 10% formalin for 24 hours. Fig. 1 Magnification×120, Fig. 2 magnification×576.

5. Biebrich scarlet, 1%, 30 seconds, or 0.25% phloxine, 15 seconds (both in 75% methyl alcohol)
6. Alcohol, 90%; 15 seconds
7. Clear, 1 minute
8. Mount in clarite

Eosin Y has also been used and has given best results with a 0.25% solution in 75% methyl alcohol. Overstaining occurs easily with this stain. In this laboratory best results have been obtained by using sections no thicker than 15 μ. Sections cut at 20–30 μ, used for biopsy evaluation, are not too thick for satisfactory results with this stain.

Discussion

Either fresh frozen sections from biopsy or fixed sections cut by the freezing method can be treated by this method. Type of fixation does not seem to affect results, and in cases where a fine differentiation is required and not obtainable with hematoxylin this stain can be used with advantage. Since over 1000 sections have not visibly deteriorated in twelve months, it can be assumed that this stain is relatively permanent.

Gallocyanin without a counterstain gives a clear nuclear picture with a faint blue background. In some tissues, such as striated muscle, the faint blue background is sufficient for a detailed study.

Malignancies stain well, cellular infiltrations are well differentiated and the nuclear pattern is well shown.

Although the method is not as rapid as the spot plate method employing hematoxylin and eosin Y (Cole, 1947), nuclear detail is better. It is not as well adapted to quick evaluation of biopsy material as the former method but for fine differentiation of cellular elements it seems to be more valuable. Time of staining employing the hematoxylin and eosin Y need not exceed 2 to 3 minutes, while in using gallocyanin 10 minutes may be required.

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STAINING OF EMBRYONIC AND SMALL MAMMALIAN SKELETAL SYSTEMS

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The methods commonly used for staining developing bone (Lundvall, 1905; Spalteholz, 1914; Batson, 1921; Dawson, 1926; Richmond and Bennett, 1938; Cumley, Crow, and Griffin, 1939; Williams, 1941), have involved the use of 1% or 2% aqueous KOH without employing any bleaching agent. The introduction of hydrogen peroxide into the technic proves to be a distinct advantage, and in the concentration here proposed, does not result in the production of fine bubbles. The maceration process can be hastened through the use of an increased concentration of potash in the absence of a fixative, although this in itself is one of the hazards of the technic. However, if the preparations are carefully watched, this difficulty may be largely avoided. The method works admirably and gives a very clear muscle tissue so that the skeletal elements are very sharply outlined. The proposed method (arranged in the form adopted in Staining Procedures) is herewith described.

Specially recommended for: Vertebrate embryos and small adult vertebrates.

Designed to bring out: Developing bone.

Method of fixation: omitted

Preparation of staining solution: Alizarin red S, (C.C.), 0.001 g.; 2% aqueous KOH, 100 ml.

Staining schedule:

1. Place fresh specimens in 5–10% aqueous KOH, to which has been added a few drops of 2% hydrogen peroxide, until the bones are clearly visible through the surrounding tissues.
   Note: If close supervision is impracticable, the specimen may be placed in 2% aqueous KOH (e.g. overnight) and then replaced in the more concentrated potash solution. If the tissues become too soft, harden 12–24 hours in a solution of equal parts glycerin, 95% alcohol, and water, and return to the clearing process.
2. Transfer to dilute (0.0025–0.01%) alizarin red S in 2% aqueous KOH until the desired degree of staining is obtained. The percentage solution is proportional to the size of the animal being stained and is greater in adult specimens.

Note: The use of acid alcohol in the case of overstaining is strongly advised against since the acid solution brings about the decalcification of minute ossification centers. If the dilution of alizarin is maintained at the proper level, overstaining will not occur.

3. Glycerin series: (72 hours)
   25 parts glycerin, 75 parts 2% aqueous KOH
   50 parts glycerin, 50 parts 2% aqueous KOH
   75 parts glycerin, 25 parts 2% aqueous KOH
   100% glycerin
4. Store in glycerin (CP) to which a crystal of thymol has been added to prevent mold.

Results: Osseous tissue—red
Soft tissues—transparent and unstained

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SIMPLIFIED PERMANENT ACETO-CARMINE SMEARS WITH A WATER-MISCIBLE MOUNTANT

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ABSTRACT.—**By employing a water-miscible mountant with a good refractive index, permanent aceto-carmine smears can be made from any of a variety of killing, fixing, and staining schedules. Clearcol, a commercial preparation, orginally developed for mounting fungi from various water-containing media, is employed for anther smears by placing the medium directly on the final stage of aceto-carmine staining schedule. Thus, no dehydration and clearing are necessary. By controlling amounts of acetic acid either mixed with Clearcol or on smears, clear, well-stained preparations can be produced.**

Ever since Belling's (1926) original work with iron-aceto-carmine preparations of anther smears for chromosome studies, there have been developed various technics for making such material permanent. McClintock's (1929) method, employing balsam or related mountants has had wide acceptance. Various mixtures of gum, resins, and other substances soluble in water, alcohols, hydrocarbons, or acids have been tried by Zirkle (1940) to reduce killing, fixing, staining, and mounting to one operation. The limitations of these combination methods are recognized by him.

The method proposed here employs a commercial preparation, Clearcol, which is miscible in water and acetic acid. It is a medium consisting of a mixture of plastics and solvents which, on evaporation of the volatile constituents, results in a clear non-crystalline preparation. According to information furnished by the originators of Clearcol, (H. W. Clark, the manufacturer[1], of Washington, D. C., and W. W. Diehl, of the U.S. Department of Agriculture), it was developed primarily for making permanent mounts of fungi without the necessity of dehydration. After the desired staining reaction has been secured from any of a number of killing, fixing, and staining schedules, the cover glass is removed (small 1/2″ or 5/8″ circles are excellent) and the Clearcol placed on the cover glass, care being taken to avoid air bubbles. The slide is then inverted over the cover glass

[1]Clearcol may be purchased on application to Mr. H. Willard Clark, 5419 32nd St., N.W., Washington 15, D.C.

and carefully pressed against the Clearcol, the whole brought right side up and the cover glass allowed to settle of its own weight. If more pressure is desired to flatten the cells, this may be applied after the cover glass has settled. In the event that the cover glass and slide are to be mounted separately to avoid different levels of focusing, a clean slide for the cover glass containing the smeared cells, and a clean cover glass for the slide with its cells may be employed.

The mountant, Clearcol, has considerable permanence. Fungi mounted with this medium have remained in an excellent state for as long as eighteen years in the mycological collections of the Bureau of Plant Industry. If any cracking of the dried Clearcol shows up near the edge of the cover glass, this can be remedied by placing a small amount of glycerin around the edge. This will be slowly absorbed by the Clearcol and retained. The refractive index is very close to balsam. It does not affect phloxine adversely after 18 years, nor does it appear to have any ill effect on carmine at the end of one year.

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STAINING NERVE FIBERS WITH METHYLENE BLUE. AN EVALUATION OF VARIABLES USED IN AN IMMERSION TECHNIC

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ABSTRACT.—**A study of the effects of osmotic pressure, pH, the presence of dextrose, acetate, pyruvate or lactate, and agents affecting cell permeability during supravital staining by methylene blue was made by means of an immersion technic. Mesentery and intestine of dogs and cats were used. Penetration of the dye was limited to the mesentery and more superficial layers of intestine. Conditions which facilitated the characteristic differentiation of of nerve fibers were: continuous oxygenation of the staining solution, pH about 5.6 stabilized by phosphate buffer, and the presence of small amounts of acetate and lactate. Young animals' tissue stained better than old. Methylene blue was a much more effective staining agent than less completely methylated thionins.**

In the experiments which form the basis of this report, an analysis was made of the factors which influence the staining of nerves supravitally by methylene blue. A series of tests on the composition of staining solutions and on the effects of variations of temperature and oxygenation were run under carefully controlled conditions. Comparable observations of the type, specificity, and density of staining reactions were made possible by the use of an immersion staining technic of thin tissues and by the utilization of a number of similar specimens from a single animal.

EXPERIMENTAL PROCEDURE

The following factors were included in the studies made on the compositon of the staining solutions: 1) osmotic pressure; 2) pH; 3) presence of dextrose, acetate, pyruvate or lactate; and 4) agents affecting cell permeability. In addition several experiments were made at different temperatures and different degrees of oxygenation.

Four-inch lengths of small intestine were removed from cat or dog under sodium pentothal anesthesia and immersed immediately into the staining solutions to be tested. Wide-mouthed specimen bottles

[1]Contribution No. 473.

(120 ml.) were used for the immersion fluid and each bottle was provided with an 8-mm. glass tube dipping to the bottom and connected to an oxygen tank through a pressure-reducing valve and distribution system to 10 bottles. The mesentery was left attached to each segment of the intestine and provided a thin tissue while the intestinal wall served as a thick one. The progress of staining was observed under a 15-power binocular dissection microscope at time intervals of adequate frequency to note changes in staining.

Nineteen dogs and eight cats were used, and an average of about eight individual staining experiments per animal performed. Representative specimens of the total 230 pieces of intestine used were saved for embedding, sectioning and further observation under higher magnification only when the results as seen with the binocular during staining seemed to justify it. Whole mounts of the mesentery were made occasionally.

Variations of the staining solutions and other variables are discussed under the individual headings which follow.

The Substrate

(*a*) *Temperature.* Trials of 26°, 37–38°, and 46°C. showed that the normal mammalian body temperature was the most effective one. Only diffuse staining wherein no nerves appeared was obtained at 46°.

(*b*) *Oxygenation.* Fresh tissue immersed in the unaerated staining solution gave only moderate selectivity for neural elements. When air or oxygen was bubbled through, selectivity was found to be much increased. Pure oxygen was more effective than air. Although it is best to differentiate tissue that has been perfused with methylene blue in a physiological saline solution without dye, the procedure of immersion first in the dye solution and afterward oxygenation in physiological saline was found to be unnecessary. Presumably, the presence of high concentrations of oxygen permits earlier and more complete oxidative differentiation. The best staining in these trials was obtained when the tissue was left in the methylene blue solution at 37–38° C., and pure oxygen bubbled through during the entire 2 to 4-hour staining period.

(*c*) *Composition of the staining solution.* Dye samples were dissolved in various salt and buffer mixtures, with and without dextrose, to test the efficacy of the carrier.

One series of tests was made to determine the effect of the tonicity of solutions. Concentrations of sodium chloride ranging from 0.5 to 2.0% were used and it was found that solutions from 0.6 to 1.5% stained with about equal effectiveness. However, solutions that were

slightly hypotonic appeared most satisfactory. It is evident that tonicity is not a critical factor in penetration or specificity of staining.

Dextrose has been advocated in many types of media to maintain metabolic activity, and was used in this study. Tests showed that the addition of 0.17 to 2.0% dextrose was of slight value in improving the quality of staining (four hour period). An addition of 0.2% was arbitrarily chosen as one which did no harm, but possibly some good, and was used subsequently.

Schabadasch (1936) and Hillarp (1946) have reported that the pH of methylene blue solutions is important in the staining reaction. The pH of solutions was altered by the addition of phosphate buffers (M/15 Na_2HPO_4 and KH_2PO_4), and checked electrometrically with a glass electrode before and after staining. Solutions ranged in pH from 5.15 to 6.9 at the beginning of staining, and the best staining occurred with solutions of pH 5.6. Five milliliters of buffer were sufficient to hold 95 ml. of staining solution at a fairly constant level (below pH 6) during a three to four hour period.

The addition of small amounts of sodium acetate has been advocated by Hillarp, and in our study it was found to facilitate staining. A series of sodium acetate solutions varying in concentration between 0.02 and 0.1% (with phosphate buffer and 0.6% sodium chloride) was tested and maximal staining found to occur with 0.03% solution.

Further experiments were run to find out whether acetate buffer had the same effect. Solutions of N/10 acetic-acid–sodium-acetate buffer (both with and without phosphate buffer) were made at pH 5.6 and added to dye solutions in varying amounts. With large volumes of buffer the sodium chloride content was reduced to keep the solution isotonic or slightly hypotonic. Acetate buffer was tried in 5, 15, 60, and 100% concentrations; it was found that 5% solution (0.005 *M*) gave the best result, but it was not quite as good as the control which contained a similar concentration of sodium acetate with phosphate buffer.

A similar series of pyruvic and lactic acid buffers were used in which the acids were neutralized to pH 5.6 with N/10 sodium hydroxide. They were mixed with dye solutions in a series of proportions representing 10 to 50% of the total volume. It was observed that only the 50% mixture of buffered lactic acid gave good staining. Acetate buffers in comparable concentration inhibited staining of nerves completely.

In another series, solutions of sodium lactate (0.5 molar) were compared with mixtures of salt, dextrose, and phosphate buffer, some with sodium acetate and others without. Solutions containing both

sodium acetate (0.03%) and sodium lactate produced more satisfactory staining than either alone. Sodium lactate seemed to improve staining of finer nerve fibers. It was found that 4 to 8 ml. of 0.5 molar sodium lactate exerted the greatest beneficial effect.

Attempts were made to alter cell permeability, to note the effect on staining by the addition of potassium, calcium, magnesium, or barium chloride (ranging from 0.03 to 1.4%), urea (0.01 to 0.20%), and glycerin (10%). All inhibited staining.

This group of experiments on the composition of the staining solution has led to the recommendation of the mixture given in the outline of technic at the end of this article.

Dye Reactions

Methylene blue (tetramethyl thionin) was used in 0.01% concentration in all of the experiments except those made with other thionin derivatives. Preliminary tests showed that higher concentrations produced more diffuse staining, and more dilute solutions gave a less clear-cut picture. The U.S.P. Medicinal form of the dye was used (National Aniline, Cert. No. 9.).

As used for immersion staining, methylene blue was found to have two phases in its reaction. Fat cells, smooth muscle, connective tissue, and blood cells always stained first. After about one hour, fine nerve fibers and then heavier ones appeared and darkened progressively until, in the typical reaction, they were more prominent than surrounding tissue. The finer fibers then tended to fade while the larger continued to darken. This apparently was due to a combination of penetration, post-mortem tissue change, and oxidation-reduction phenomena. The cause of the rather indifferent staining of smooth muscle, connective tissue, and fat cells was thought to be due to the presence of small amounts of demethylated methylene blue (dimethyl and trimethyl thionin) in the solution. It has been observed that old methylene blue solutions or fresh solutions oxidized with peroxide do not stain nerve fibers, and to verify the effect of demethylation on staining, a series of experiments was run using the azures and thionin.

Samples of trimethyl thionin (azure B), dimethyl thionin (azure A), monomethyl thionin (azure C), and thionin (Lauth's violet) were used in 0.01% concentration in a buffer at pH 5.6 to observe their reaction with the various tissue elements. Controls of methylene blue were run in each case.

Azures A and B stained nerve fibers with about half the intensity of methylene blue controls, while very faint staining occurred with azure C and thionin. The protoplasm of fat cells and smooth muscle

fibers stained intensely with each dye. The color produced ranged from violet to pink, being darkest with azures A and B and lightest with thionin. Solutions of the azures were different from fresh methylene blue in being redder in tone. Methylene blue solutions which stand for more than three or four days take on this red coloration which is apparently due to conversion of the tetramethyl to the tri-, di-, and monomethyl compounds. Such solutions should not be used in staining of nerve fibers.

Holmes (1928) showed by means of spectrophotometric analyses that commercially prepared methylene blue contains about 10% trimethyl thionin. Our studies suggest that this contaminant may be, at least in part, responsible for the staining of elements other than nerve fibers. Both Holmes, and Lillie (1944) prepared nearly pure methylene blue but neither used the material for supra-vital staining. Work is in progress dealing with the purification of methylene blue in attempt to obtain more specific staining.

The Experimental Animal

Early in the course of this study, it became obvious that the nerve tissue of no two animals reacted identically. As a general rule very young animals have tissue which stains well; adult animals have a moderate ability to stain, and old animals frequently have nerves which stain very poorly or not at all. Our data do not permit an evaluation of the causes of such variations, but the role of methylene blue as a hydrogen acceptor in carbohydrate metabolism should be considered in relation to the nutritional state and the aging process. Nutritional experiments might shed light on the problem.

At this time it can be said that nerve fibers from a freshly killed young cat or dog will nearly always stain well. Staining reactions were graded arbitrarily from 0 (with no nerve staining) to 4 (most complete and best differentiated). Table 1 shows the results obtained.

Table 1. Comparison of Staining Reactions of Young and Old Animals.

Grade of stain	0	1	2	3	4
Young dogs	0	2	1	4	7
Old dogs	1	1	2	1	0

Although the series is too small for conclusions, tissue from young animals seems to be preferable to that from old.

If the animal has been dead for over four hours, staining is usually unsatisfactory; though if the tissues are kept on ice one may occa-

sionally obtain good staining as long as ten hours after death. The average time limit, however, is two to three hours.

Preservation of Specimens

(*a*) *Fixation.* The preservation of the stained tissues involves several problems. Fixation must be rapid to prevent fading, and such that the dye will not dissolve out during dehydration and clearing. Eight per cent ammonium molybdate (a commonly used reagent) produces a fairly insoluble precipitate of methylene blue but is a poor tissue fixative. Poljak (1941) has developed a number of improved fixing fluids, by combining formalin or dichromate and either acetic or trichloracetic acid with the molybdate. We have used a modification of his molybdate, dichromate formula without acid. One per cent potassium dichromate was found to be compatible with 8% ammonium molybdate for at least 24 hours and gave better fixation than molybdate alone. After 24 hours this mixture may precipitate, but tissues that can be stained by immersion would not require more time than this. After a 24-hour fixation, tissues were washed for a similar length of time in several changes of distilled water.

(*b*) *Dehydration.* Molybdate-precipitated methylene blue is partly soluble in the usual alcohols used for dehydration. Tertiary butyl alcohol (recommended by Levine, 1939) has only a very slight solvent action and was a satisfactory dehydrating agent. It has the disadvantage of penetrating about half as fast as ethyl alcohol so that dehydration takes longer. Segments of bowel required four to five days treatment before dehydration was complete. Another disadvantage is that it has a melting point of 25.5° C., and must be kept above this temperature to prevent crystallization.

Specimens were transferred from the wash water to full strength tertiary butyl alcohol after the excess water was removed with blotting paper. The alcohol was changed twice during the course of dehydration. Clearing with xylene and embedding in paraffin were handled as with ordinary preparations, or whole mounts in clarite were made.

Summary

Many reports have been written about the staining of nerve fibers with methylene blue, and each author has advocated certain refinements in technic which have tended to make the procedure increasingly complex. The impression is gained that each modification will yield constant results and yet, in our hands, this has not been true. This study was initiated to isolate and evaluate as far as possible the factors involved.

The methylene blue reaction is characterized by, first, the absorption of the dye with concommitant reduction to the leuco-base and, second, the oxidation of the colorless form of the dye to produce the stain. Whether the dye is reduced to the colorless form depends on the oxygen tension or reducing capacity of a given tissue. During staining by immersion, the superficial structures stain first, since they are in more intimate contact with both dye and oxygen than deeper ones. The first phase of staining is not visibly specific for nerve fibers, but it seems probable that they are concentrating the dye and simultaneously converting it to the colorless reduced form. Later, as their vital processes lag, the oxidation of the dye reveals them more intensely stained than the surrounding tissues. With immersion staining, differentiation of nerve fibers progresses in the presence of the dye, and it is not necessary to differentiate in a solution which contains no dye as has been recommended for perfused specimens. Differentiation appears to depend upon the physiological state of the tissue and the presence of oxygen.

The physiological state of the nervous system is so variable that no two animals react alike to methylene blue as used in present methods. This makes exact repetition of experiments difficult or even impossible. In spite of this, one occasionally obtains very beautiful preparations which clearly demonstrate the architecture. If young, well nourished healthy animals are used, the results are more satisfactory than with older animals.

Important technical factors in immersion staining appear to be the use of the following:

1. Fresh methylene blue solutions.
2. Temperature 37–38° C. (for mammals).
3. Solutions kept saturated with oxygen.
4. pH between 5 and 6, preferably 5.6.
5. Isotonic or slightly hypotonic solutions.

Suggested outline of immersion technic.

1. Young, healthy mammals are killed with pentobarbital.
2. Thin tissues are immersed in:

Methylene blue (U.S.P. medicinal)	0.01 g.
Sodium chloride	0.60
Dextrose, C.P.	0.20
Sodium acetate	0.03
Distilled water	90.00 ml.
Phosphate buffer, M/15, at pH 5.6	5.00
Sodium lactate, M/2	5.00

Add the buffer and lactate after the methylene blue has dissolved.

3. Keep the staining solution at 37–38° C. and allow a small stream of pure oxygen to bubble through it during the course of staining.
4. Observe the specimens at 0.5-hr. intervals with 10 × or higher magnification.
5. When the differentiation of nerve fibers appears to be optimum (2–4 hr.) fix in:

   Ammonium molybdate ..... 8.0 g.
   Potassium dichromate 1.0
   Distilled water ..... 100.0 ml.

   Renew the solution after 24 hr. if longer fixation is desired.
6. Wash with several changes of distilled water for 24 hr.
7. Dehydrate in pure tertiary butyl alcohol (at 26–28° C.) for 3 to 5 days and change the alcohol twice.
8. Clear in zylene. Prepare whole mounts with clarite or embed in paraffin and section.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

BARRON, A. L. E. **Microscope mirrors.** *Microscope*, **6**, 113–4. 1947.

Suggestions for improvements in microscope lighting are made. To achieve a single reflection of the light source, reflection from a first surface mirror made of aluminum vacuum-deposited on glass is recommended. Correct alignment in which the center of the mirror coincides with the optic axis also greatly improves lighting and is easy to attain.—*C. Randall.*

COLLINS, W. G. **Process timer.** *Microscope*, **6**, 115. 1947.

The author describes a timer which can be set for any internal up to 35 min. with an accuracy of plus or minus 2 sec. The use of a small synchronous motor, such as those used to drive industrial recording instruments, is the basis of the design.—*C. Randall.*

KESSEL, ALBERT M. **Multiple tray for embedding tissues in paraffin.** *J. Nat. Cancer Inst.*, **5**, 257–9. 1945.

The author describes an excellent device for mass embedding, consisting of a multiple paraffin embedding tray which has 5 units each containing 10 compartments. Each compartment holds 2 or 3 paraffin blocks depending on the size of the tissues and is separated from its neighbor by easily removable metal strips. The metal parts drop off when lightly tapped after cooling. A complete description of the construction is given.—*Paul G. Roofe.*

SCOTT, T. L. **Slide Rack.** *Microscope*, **6**, 122–4. 1947.

Details are given for the construction of a rack measuring approximately 4×3¼×2¼ in. Essentially it consists of 2 plates held apart by threaded rods. The plates are provided with arms which support the slides. Two other slide rack designs are also given.—*C. Randall.*

MICROTECHNIC IN GENERAL

KUPPERMAN, H. S., and NOBACK, C. R. **A rapid iron hematoxylin stain utilizing a combined fixative-mordant solution.** *Arch. Path.*, **40**, 78. 1945.

The addition of 1% ferric ammonium sulfate to Bouin's solution, (U. S. P. formalin 1:10 containing 0.5% acetic acid) and Lavdowsky's mixture (U. S. P. formaldehyde, 10 ml., 95% ethanol, 50 ml., glacial acetic acid, 2 ml., and distilled water, 40 ml.) gave a combined fixing-mordanting solution which is recommended for decreasing the time required for the Heidenhain staining procedure.

Tissues are fixed and mordanted in any of the above solutions for 1–3 days, depending on their size. They are then dehydrated in ethanol or dioxane for paraffin infiltration, and tissue sections after deparaffination are stained directly in Heidenhain's hematoxylin solution, destained with saturated aqueous picric acid, then counterstained, dehydrated, cleared and mounted in clarite or balsam. —*L. Farber.*

STEINHERZ, A. R. **The shape of particles in finely ground powders.** *J. Soc. Chem. Ind.*, **65**, 314–20. 1946.

Three methods for determining the particle size in the sub-sieve range are discussed. For microscopic counting and measurements, suspensions are pre-

pared of dry cement and glass particles in ethylene glycol, containing 1 mg. sodium citrate per ml. as an anticoagulating agent. Particle counting is carried out with a Thoma counting chamber and an eyepiece micrometer-net.—*R. T. Whittenberger.*

DYES AND THEIR BIOLOGICAL USES

CORRIN, M. L., and HARKINS, W. D. **Determination of the critical concentration for micelle formation in solutions of colloidal electrolytes by the spectral change of a dye.** *J. Amer. Chem. Soc.*, **69**, 679–83. 1947.

The suitability of a dye as an indicator in the determination of the critical concentration for the formation of micelles in soap solutions is related to the existence of an equilibrium mixture of the dye in aqueous solution from which one form is preferentially solubilized by the soap micelle. The charge on the dye ion must be opposite to that of the micelle. In general, the variation of the spectrum of a dye with change in environment is not a sufficient condition for the suitability of the dye as a critical concentration indicator. Pinacyanol chloride and rhodamine 6 G are shown to be suitable for the determination of the critical concentrations of anionic soaps, while sky blue FF, eosin, fluorescein, and acidified 2,6–dichlorophenolindophenol may be used with cationic detergents. The necessary presence of acids, bases or buffering salts renders the use of acid-base dyes less satisfactory than dyes in which a polymerization equilibrium exists. The critical concentration may be determined by a titration in which the disappearance of micelles is determined by a sharp change in color or by a sharp reduction of fluorescence. Certain dyes exhibit both of these changes.—*R. T. Whittenberger.*

FISCHER, E., and MUNOZ, R. **Comparative bacteriostatic assays with rosaniline and its phenol analog (rosolic acid).** *J. Bact.*, **53**, 381–8. 1947.

Rosaniline and rosolic acid are bacteriostatic at fairly high concentrations. The rosaniline is more effective in alkaline medium than in neutral or acid; the rosolic acid is equally effective at the various pH values tested. Although the lot of rosaniline used was not specified, the rosolic acid was prepared from the same sample of dye.—*V. Kavanagh.*

TILLEARD, D. L., and SMITH, N. D. P. **Comparison of optical and electron microscopy of pigments.** *J. Soc. Chem. Ind.*, **65**, 261–4. 1946.

Optical and electron micrographs of identical pigments are shown. In the electron micrograph a red earth pigment is seen to contain many fine, almost transparent particles which by reason of their small size are not visible in the optical micrograph. In the electron micrograph of zinc oxide, particles are resolved whose shape is undisclosed in the optical micrograph. An important advantage of the electron microscope over the light microscope is that a great depth of focus is obtainable at high magnifications. Caution must be taken in obtaining quantitative data on particle size distribution with the electron microscope, since the field of view is small and large particles may be missed.—*R. T. Whittenberger.*

ANIMAL MICROTECHNIC

BAILLIF, RALPH N., and KIMBROUGH, CECIL. **Studies on leucocyte granules after staining with Sudan black and May Grünwald Giemsa.** *J. Lab. & Clin. Med.*, **32**, 155–66. 1947.

Dried blood films can be advantageously studied after staining as follows: Fix for 5 sec. in a mixture of 1 volume of formalin and 9 of 95% alcohol, wash in distilled water and dry. Stain with Sudan black B (70% alcohol saturated with the dye) for 30–60 min. at room temperature. (The colors will be darker at 37° C.). Flood the slide with May-Grünwald mixture for 3 min. and cover with neutral distilled water for 1 min. Drain without washing and cover with dilute Giemsa mixture (1 drop stock solution to 2 ml. of water), for 15 min. Wash in distilled water and dry. Erythrocytes are stained slate gray to black. Neutrophile granules are small spherical bodies collected in groups in the cytoplasm. Eosinophilic granules are larger and composed of a peripheral deeply sudanophile shell with a central sudanophobic core. Basophilic granules and azurophile elements are sudanophobic. Oxidase and peroxidase granules are probably always sudanophilic.—*John T. Myers.*

BHATTACHARJI, L. M., SINGH, JASWANT, and SEN GUPTA, G. P. **A simple methylene blue-eosin substitute for Leishman and Giemsa stains.** *Indian Med. Gaz.*, **81**, 400–1. 1946.

War shortages necessitated finding a substitute for Leishman and Giemsa stains. After experimenting with different concentrations of dyes and oxidizing agents, the results achieved with the following formula were found to be as good as with any of the standard stains. Preparation of stain: Dissolve 1.0 g. methylene blue in 100 ml. H_2O and 0.3 g. $KMnO_4$ in 100 ml. water. (Water used need not be distilled, for slightly alkaline tap water with a pH value of 7.2 to 7.6 is satisfactory.) Transfer the methylene blue solution to a procelain dish and heat so it steams but does not boil for 5 min. Gradually add the $KMnO_4$ solution. (A fine layer of crystals form on the surface.) Continue heating for 10 min. Dissolve 0.4 g. eosin in 50 ml. water and add to steaming mixture, stirring with a glass rod, while a thick yellow scum appears. Continue heating, not too strongly, for an hour or until the solution has evaporated, leaving a thick scum with metallic luster, at first copper red and when dry, green. (The residue should be left overnight in a desiccator or incubator at 37° C. When completely dry the scum will peel off as shining green flakes. If necessary remove with a scalpel.) Powder the flakes. in a dry glass mortar and store in a dry air-tight glass container. As a substitute for Leishman stain put 0.1 g. of the powder in a glass mortar and dissolve thoroughly by grinding it while adding 40 ml. methyl alcohol in small quantities at a time. Transfer to a small bottle and use for staining blood smears, both thick and thin, by following Leishman's method. As a substitute for Giemsa stain, put 0.3 g. of powder in a glass mortar and grind it while very gradually adding a mixture of glycerin and methyl alcohol (25 ml. each). Transfer this solution to a small glass bottle and keep at room temperature until the next day, then place bottle neck deep in a H_2O bath for 2 hr. Follow Giemsa's method for staining.—*G. N. Ludwig.* (Courtesy *Biological Abstracts*).

DEANE, HELEN W., NESBETT, FRANCES B., and HASTINGS, A. BAIRD. **Improved fixation for histological demonstration of glycogen and comparison with chemical determination in the liver.** *Proc. Soc. Exp. Biol. & Med.*, **63**, 401–6. 1946.

Rossman's fixative (neutral formalin, 1 part; picric acid, saturated solution in absolute alcohol, 9 parts,) used at 0° C. preserved glycogen in rat liver uniformly throughout the block. The low temperature of fixation gave sections which were uniformly stained, without the usual rim of more intensely stained cells seen after fixation at room temperature. Glycogen granules were distributed throughout the cytoplasm of liver cells rather than concentrated against one membrane. Quantitative determinations of glycogen were made by chemical means and by photometric measurements on the stained histological sections. Comparisons of values obtained showed excellent agreement. Glycogen was stained by the Bauer-Feulgen reagent (See Bensley, C. M., Stain Techn., **14**, 47, 1939). Four photomicrographs show the optical density and other characteristics of the stain.—*H. A. Davenport.*

DISCOMBE, G. **The nature of neutrophilic granulation.** *J. Path. & Bact.*, **58**, 572–3. 1946.

As recognized earlier by Lison, the granules of neutrophils and eosinophils are probably lipoid. They stain with Sudan black in a manner indistinguishable from that seen in good oxidase preparations. The reason for their staining with Leishman's stain is that one of the components of the stain is adsorbed on the surface of the lipoid granule. This component is probably Bernthsen's violet since a simple aqueous solution of this basic dye stains granules well.—*S. H. Hutner.*

DIGGS, L. W. and SURRIDGE, MARJORIE S. **A study of the sternal marrow and peripheral blood of fifty-five patients with plasma cell myeloma.** *J. Lab. & Clin. Med.*, **32**, 167–77. 1947.

Films from sternal marrow stained with Wright stain showed increased cellularity; relative and absolute increase in plasma cells of 4 to 90% (more than 10% in most cases), increase in the number of plasmoblasts and early plasma cells in mitosis; grouped plasma cells; nuclear abnormalities in plasma cells consisting of multiple nuclei, nuclear fragments, and indented and lobulated nuclei; cytoplas-

mic abnormalities consisting of displacement or absence of unstained areas, marked variation in size and shape with projections and ragged streamers; relative and absolute decrease in myeloid, erythroid and megakaryocytic elements, and a tendency toward immaturity. This supports the view that plasma cells arise from reticulum, that they constitute a distinct strain of cells, and that there is no sharp line of demarcation between solitary plasmacytoma, plasma cell myeloma, and plasma cell leukemia.—*John T. Myers.*

ELLIOTT, K. A. C. **Swelling of brain slices and the permeability of brain cells to glucose.** *Proc. Soc. Exp. Biol. & Med.*, **63**, 234–6. 1946.

Slices of rat brain cortex swell in isotonic saline more than do similar slices of liver. Brain slices swell in an electrolyte-free immersion fluid of 2× isotonic glucose, fructose or sucrose (25–58%). Such swelling is prevented by the addition of electrolytes, 0.2–0.4× isotonic, to 1.6× isotonic sugar solutions. Author concludes: "Brain tissue behaves as if freely permeable to glucose, fructose and sucrose in the absence of electrolyte but, in the presence of small amounts of salt, these sugars behave as impermeable solutes." Swelling was minimal in Ringer's solution plus 3% gelatin, in blood serum, in isotonic $CaCl_2$ and in isotonic $MgCl_2$. —*H. A. Davenport.*

GOMORI, GEORGE. **Staining of chromaffin tissue.** *Amer. J. Clin. Path., Tech. Sect.*, **10**, 115. 1946.

Heidenhain's azocarmine modification of Mallory's aniline blue collagen stain is suggested as a satisfactory specific stain for chromaffin tissue. The following procedure is recommended for paraffin sections fixed in aqueous non-dichromate fixative solution, rather than in alcoholic fixatives:

Remove paraffin in xylene and pass through ethanol to water; stain 60–90 min. at 56–60° C. in 0.05% solution of azocarmine G in 1% acetic acid; rinse in tap water, blot and rinse in 95% ethanol; differentiate under microscopic control in 1% solution of aniline in 90–95% ethanol to point where chromaffin cells are a deep pink shade against paler cortical cells, requiring 5–60 min. depending on type of fixation and thickness of section; rinse in tap water; immerse 20 min. in 2–3% solution of phosphotungstic acid; wash 1 min. in tap water; stain 15–40 min. in Mallory's mixture of aniline blue and orange G (quinoline yellow or tartrazine may be substituted for orange G with better contrast) until connective tissue is deep blue; rinse in tap water; dehydrate, clear and mount.

Chromaffin granules of human adrenal medulla stain bright purplish red against a dull orange (or greenish yellow using quinoline yellow or tartrazine) of cortical cells. Rat and rabbit chromaffin tissue stain a more bluish purple color. In tumors chromaffin granules range in color from bright ruby red to almost clear blue.

Granules in alpha cells of pancreatic islets, and in some cells of anterior pituitary, granulations of human neutrophil leucocytes and of fresh myelocytes and granules of entero-chromaffin cells of guinea pig and man stain similarly to those of chromaffin tissue of adrenal medulla.—*L. Farber.*

HIGHMAN, B. **Improved methods for demonstrating amyloid in paraffin sections.** *Arch. Path.*, **41**, 559. 1946.

Amyloid in paraffin sections fixed in formaldehyde may be demonstrated satisfactorily by either of the following methods:

(1) Deparaffin in xylene, and pass through graded alcohols to water; stain 5 min. by Weigert's iron-hematoxylin-acid method; wash in water; stain 1–5 min. in 0.5% crystal violet or methyl violet in 2.5% acetic acid; wash in water and mount in modified Apathy's syrup prepared by dissolving 50 g. each of gum acacia and cane sugar and 10 g. of NaCl in 100 ml. of water with 1.5 ml. of 1% merthiolate as preservative. Amyloid, cartilage and some types of mucoid material stain purplish to violet red against a bluish background. Nuclei are sharply stained by the hematoxylin.

(2) Deparaffin; stain 1–5 min. (until deep red) in 0.5% Congo red or Congo corinth G (Colour Index No. 375) in 50% ethanol; wash in water; differentiate 1–3 min. in 80% ethanol containing 0.2% KOH; rinse in water; counterstain with iron or alum hematoxylin; wash in water; dehydrate, clear and mount by successive use of acetone, acetone-xylene, xylene and clarite.—*L. Farber.*

LERNER, EDWIN M. A rapid Gram stain for tissue. *Arch. Path.*, **41, 674. 1946.**

Tissue, fixed 24 hours in Zenker's Solution and embedded in paraffin, is sectioned at 6 μ, mounted on slides, and heated at 56°C. for 2–6 hours. The tissue sections are then stained as follows:

Treat 10 min. with xylene, 10 min. with absolute ethanol and rinse in 95% ethanol; stain 1 min. in Sterling's gentian violet solution (5 g. crystal violet, 10 ml. absolute ethanol, 2 ml. aniline, 88 ml. distilled water); wash in water; treat 1 min. with iodine solution (1% iodine in 2% aqueous KI); wash in water, decolorize 10–30 sec. with 1:1 mixture of acetone and absolute ethanol; wash in water; counterstain 30 sec. with 1% aqueous safranin solution; wash in water; rinse in 95% ethanol to remove excess safranin; rinse in absolute ethanol and xylene; mount in balsam.

Gram-positive organisms are stained deep blue to violet; Gram negative organisms, red; nuclei, pink to red, with good detail; cytoplasm, light yellowish pink. Bacterial spores appear as clear spaces within cells.—*L. Farber.*

PROESCHER, F., ZAPATA, E. M., and MC NAUGHT, J. B. A rapid method of staining tissue sections for immediate diagnosis. *Amer. J. Clin. Path., Tech. Sect.*, **10, 50. 1946.**

The staining method is similar to the hematoxylin-eosin technic in results, but differs mainly in specifying azophloxine (protoplasmic stain), celestin blue (nuclear stain) and euparol (for clearing and mounting).

Staining solutions used are as follows: 1) 0.1% aqueous azophloxine (CI No. 31); 2) an aqueous solution containing 1.0% celestin blue (CI No. 900), 10.0% ferric ammonium sulfate, 14% by vol. glycerol and 2% by vol. H_2SO_4. This is prepared by the following procedure: add the celestin blue and ferric alum to the water; boil 5 min. with constant stirring; cool, filter and add the glycerol and H_2SO_4; make up to volume. Euparol is a mixture of comsol, eucalyptol, paraldehyde and sandarac with a refractive index of 1.43 and is obtainable from Arthur H. Thomas Company of Philadelphia.

The following procedures are recommended:

Fixation of tissue—place 5 mm. thick piece of tissue in a 50 ml. Pyrex test-tube containing 15–20 ml. of 10% formalin solution and boil over an open flame for 30 sec.; pour into small basin of water, from which wet tissue is placed on block holder of microtome and partially frozen.

Sectioning—section tissue, just before it is frozen hard to avoid brittleness, 15 μ thick, remove from knife with wet finger, place in small basin of tap water, dip up a flat intact section in a small wire-gauze basket for sectioning. Wire basket is made by forming a circular 2.5 cm. loop of stiff copper wire, leaving a 3 cm. handle at right angle to the loop and soldering the free end to complete the circle; a fine bronze wire gauze is soldered about the bottom of the circle forming a basket.

Staining—prepare stain just before use by mixing solutions 1 and 2 in proportion of 2:1 and pour into a Syracuse type of watchglass. Place the basket with wet section in the stain for 10–30 sec., then in basin of tap water so that stained section is freed from basket, pick up section on glass slide and blot free of water with dry cloth. The addition of 1–2 drops of 95% ethyl alcohol on stained section in basket before dipping into water makes it flatten out more easily.

Dehydration—Flood slide with 95% ethyl alcohol and drain, flood with aboslute alcohol and drain, place drop of euparol on middle of section, cover with cover slip and examine.

Sections are permanent and comparable with results obtained by paraffin procedure using hematoxylin and eosin, but with less marked shrinkage.—*L. Farber.*

ROMANES, J. G. A new silver method for staining paraffin sections of the nervous system. *J. Anat.*, **80, 205–7. 1946.**

At Cambridge only Bayer's pre-war protargol has proved satisfactory for Bodian's (1936) method for staining paraffin sections of nervous tissue. Because of the difficulty in obtaining this substance, another method, using colloidal silver solutions, was sought.

The method devised is as follows: Fix nervous tissue in neutral formalin. Pass sections through benzene; embed in paraffin. Attach to slides with albumin. It is recommended that the sections be floated on alcohol (70%), blotted with cigarette paper, and dried on a hot plate. Bring sections to water, tap or distilled, then place in the impregnating fluid for 4–24 hr. at 58° C. Rinse rapidly in water. Develop for 5 min. in: Pyrogallol, 1 g.; hydroquinone, 1 g.; sodium sulphite cryst, 10 g.; distilled water 100 ml. Wash well in water. Stages 1–4 may be repeated if more intense impregnation is required. Then transfer to 0.3% gold chloride in 2% glacial acetic acid for 5 min., rinse in distilled water, and leave 10–15 min. in 2% oxalic acid in 1% formalin. Wash well, treat with 5% hypo, 5 min., dehydrate, clear and cover.

The impregnating solution is prepared as follows: Dissolve 1 g. of $AgNO_3$ in 20 ml. of distilled water and just enough dilute ammonia to dissolve the ppt. formed by the first few drops. Dilute to 1 l. with distilled water. This forms a stock solution. To 30 ml. of above solution add 10 ml. of 1% gelatin in fluid condition (slightly warmed), 0.8 ml. of 0.5% tannic acid in distilled water and 0.3 ml. of pure pyridine, stirring the solution all the time. This solution, which gradually grows dark brown due to formation of colloidal silver, should be made up fresh shortly before use and discarded after impregnation.

After correct impregnation the sections show purplish-black fibers on a clear background and reddish cells with well-marked neurofibrils. Boutons are not well shown. With cerebral cortex, 10% chloral hydrate should be added to the 4% formaldehyde used for fixation.—*Warren Andrew.*

STENGER, ALBERT H., and CHARIPPER, HARRY A. **A study of adrenal cortical tissue in Rana pipiens with special reference to metamorphosis.** *J. Morph.*, **78**, 27–42. 1946.

For studies on the adrenal cortex of the frog during several stages of metamorphosis, Bouin's, Flemming's (the stronger fluid without acetic acid), Champy's and Helly's fluids were used as fixatives. The last two solutions were of special value in identifying adrenal cortical tissue, because of the blackening action of the osmium on the lipoid granules of the cortical cells. Post-chromation in 3% aqueous potassium bichromate, or use of the Champy-Kull technic, facilitated identification of chromatin cells.

Dawson and Friedgood's azocarmine technic after Champy or Flemming fixation and post-chromation proved most satisfactory. Early cortical cells were stained red, late ones blue; Stilling cells a brilliant carmine; cells of the medullary adrenal orange or brown.—*Elbert C Cole.*

ZORZOLI, ANITA. **Effects of vital dyes on early development of the amphibian embryo.** *Proc. Soc. Exp. Biol. & Med.*, **63**, 565–9. 1946.

Nile blue sulfate, neutral red and Bismark brown in concentrations of 1:10,000 to 1:1,000,000 were studied for their effects on developing eggs of *Rana pipiens.* Critical concentrations, below which normal development occurred were: N. B. S., 1:750,000; N. R., 1:250,000; B. B., 1:100,000. The gastrulation stage was most vulnerable, with stoppage of growth or lag of the animal hemisphere with the result that masses of yolk material remained exposed. Eggs in the 2-cell stage were exposed for timed intervals (3–67 hr.) in graded dye concentration and then were transferred to fresh spring water. Normal development followed by abnormal was seen at gastrulation. Exposure of embryos at various stages of development showed maximum effects at stages 9 and 10, decreasing at 11 and 12, and no abnormalities produced at stage 14. Dye solutions buffered to pH 4.5 were without effect while those of pH 6.3 to 8.5 gave the same results as unbuffered dyes.—*H. A. Davenport.*

PLANT MICROTECHNIC

JONES, RICHARD C. **The Feulgen reaction as a cytological technique with Allomyces arbuscula.** *Mycologia*, **39**, 109–12. 1947.

Young plants of *Allomyces arbuscula* were fixed in each of four fixatives for varying lengths of time. The fixatives used were: (1) Feulgen's, (2) Carnoy's A, (3) Carnoy's B, and (4) a Meve's modification of Flemming's solution. Formulae

and a summary of staining technic are included in the article. The following procedure was found to give satisfactory results: fixation in Feulgen's or Carnoy's B; hydrolosis; 5–20 hr. staining in fresh basic fuchsin solution; bleaching in three dips of Johansen's aqueous solution of SO_2; dehydration in an alcohol series; clearing in xylene; mounting in Canada balsam.—*Elbert C. Cole.*

MICROÖRGANISMS

DIERCKS, F. H., and TIBBS, R. O. **A rapid method for the staining of Rickettsia orientalis.** *J. Bact.*, **53**, 479, 480. 1947.

McNeal's tetrachrome stain, prepared according to the directions of the manufacturer, has been used for routine staining of air-dried smears of yolk-sac material infected with Rickettsia. The method is as follows: Fix and kill in absolute methyl alcohol for 3–5 min.; prepare dye solution by adding 4–5 ml. of stock solution to 75 ml. distilled water; transfer slides directly from fixative to stain with gentle agitation and leave for 15–20 min.; clear by dipping in absolute acetone until stain no longer dissolves; blot dry; examine under oil-immersion lens using a blue ground-glass filter. The background is pinkish; Rickettsia are dull blue; bacterial contaminants are bright blue; and leucocytes and erythrocytes are differentially stained. This method is not as good as the one employing Giemsa stain for photographic work but is faster and gives more consistent results when used by ordinary laboratory helpers. It is not recommended for use on animal tissue smears.—*V. Kavanagh.*

DYAR, M. T. **A cell wall stain employing a cationic surface-active agent as a mordant.** *J. Bact.*, **53**, 498. 1947.

The cell wall of bacteria and yeasts was stained as follows: Heat-fix a smear; add 3 drops M/100 (0.34%) cetyl pyridinium chloride; add 1 drop saturated aqueous Congo red, mixing on slide; wash; counterstain with methylene blue (no specifications given); wash; examine in water under a cover slip or in oil. The cell wall is red, the cytoplasm is blue.—*V. Kavanagh.*

HENDERSON-BEGG, A., and FULTON, FORREST. **The standardization of a scrub typhus vaccine.** *J. Path. & Bact.*, **58**, 381–9. 1946.

For a quick routine staining of rickettsiae the following procedure may be used: Fix light impression smears of the cut surface of lung with gentle heat. Treat with *N* HCl, 2 min. (this enhances stainability with basic dyes): wash and stain 30–60 min., with 1:2000 polychrome methylene blue (polychromed by heating 45–60 min. at 56°C. with 1% Na_2CO_3; on cooling neutralized with *N* HCl and filtered; diluted 1:20 before use).

To stain rickettsiae a different color from cells, prepare smears of uniform thickness and free from blood. Fix with gentle heat and treat for 10–15 sec. with 10% HCl. Wash and stain 2 min. with boiling carbol fuchsin (Ziehl-Neelsen diluted 1:5); and wash. Differentiate and counterstain with 1% malachite green, 30–60 sec. (This stage is critical.) Rickettsiae stain deep reddish purple against a greenish blue background of cytoplasm and nucleus. The same method is suitable for material fixed in formol-Muller; after staining with boiling carbol-fuchsin, the sections are decolorized 10–15 sec. with 5% citric acid and counterstained 10–25 sec. with malachite green.

To determine the rickettsial content of vaccine, stainable rickettsiae are counted by the following technic. Mix a small amount of vaccine with an equal volume of a formalin-killed suspension of *Clostridium welchii* of accurately determined concentration (homogenized by spraying through an atomizer). Dry the mixture on a slide; fix the dry film with heat. Treat 5–10 min. with 40% formalin, wash and stain 2 min. with boiling carbol-fuchsin. Wash; decolorize 10–15 sec. with 5% citric acid. Counterstain 10–25 sec. with 1% malachite green. Rickettsiae stain red, *Cl. welchii* deep crimson and the background pale greenish blue. The rickettsiae are generally clumped, hence for the most accurate counts the clumps should be broken up by spraying; this method is too tedious for routine use.—*S. H. Hutner.*

JOANNIDES, GEORGE. **A simple method of staining malaria parasites.** *J. Lab. & Clin. Med.*, **32**, 89. 1947.

The following method is satisfactory for staining malarial parasites: Place 0.5 g. methylene blue and 1.5 g. borax in a 50 ml. porcelain dish. Add 30 ml. of boiling tap water with stirring, place over a small flame and evaporate almost to dryness at 75–80° C. Repeat this twice. Let stand at room temperature 24 hr.; without stirring add 30 ml. of 95% alcohol. After 5 minutes pour the alcohol into a 100 ml. bottle and let the residue dry almost completely. Repeat the extraction once with 30 and once with 40 ml. of alcohol. Place 15 drops of this alcoholic stain on an unfixed blood film. After 30–60 sec., add 15 drops of a 1–2000 solution of eosin in tap water, and mix well with a glass rod. After 3–4 min. flood with tap water, wash, and let dry. Red cells are light green, slightly pinkish; eosinophilic granules are pink; the remaining cells and malarial parasites stain as with Giemsa stain.—*John T. Myers.*

LILLIE, R. D. **Reactions of varous parasitic organisms in tissues to the Bauer, Feulgen, Gram, and Gram-Weigert methods.** *J. Lab. & Clin. Med.*, **32**, 76–88. 1947.

Sharply stained nuclei were demonstrated by the Feulgen method in the following: *Sarcosporidia, Toroplasma, Klossiella muris, Trypanosoma cruzi, Plasmodium gallinaceum,* in eggs, larva and adults of *Schistosoma japonicum* and *S. mansoni*; in larvae and adults of tapeworms, round worms, and *Onchocerca volvulus,* and in eggs and larvae of *Capillaria hepatica.* Poorly stained or dubiously Feulgen positive nuclei were encountered in Encephalitozoon, *Eimeria stiedae, Endameba histolytica, Torula histolytica, Blastomyces,* and *Histoplasma capsulatum.* The mycelial fungi, bacteria, Rickettsiae, *Coccidiodes immitis,* and *Haplosporangium parvenum* failed to present Feulgen staining material. The Weigert-anilin-Gram technic gave more Gram positive organisms than did the Gram-acetone method. Bauer-positive polysaccharides digestible with ptyalin and with malt extract form the capsules of *Endameba histolytica, Eimeria stiedae,* most Sarcosporidia, and are present in the egg cytoplasm of *Capillaria hepatica.* Bauer-positive material is also present in *Monilia candida, Histoplasma capsulatum, Torula histolytica, Haplosporangium parvenum, Coccidoides immitis,* and *Blastomyces, Mycetoma,* certain coarse mycelial fungi as *Penicillium* and *Aspergillus,* the shell of the eggs of *Schistosoma,* and the opercular plugs of *Capillaria hepatica,* also in some Sarcosporidia, and a few individuals of *Klossiella muris,* and in *Toxoplasma.* The Bauer method is valuable in detecting scarce amebae and yeasts in tissue.—*John T. Myers.*

PIJPER, A. **Shape and motility of bacteria.** *J. Path. & Bact.*, **58**, 325–42. 1946.

This paper propounds the revolutionary thesis that propulsion in bacteria is by spiral movements of the cell and that flagella are somewhat accidental passive appendages. Most of these observations were carried out by means of dark-field microscopy using sunlight as light source.

Bacteria may be slowed for observation by means of methylcellulose ("methocel", Dow Chemical Co.). The method is as follows: Prepare graded solutions (1.5 to 2%) of methylcellullose and add a drop to a slide using a 5 mm. loop. Gently rub on a drop from a 1 mm. platinum loop taken from a very motile broth culture. Add a cover slip and seal. The 4000 centipoise type of methocel appeared the most useful. Unlike gelatin and gums, methocel does not precipitate on bacteria or produce artifacts.

The author believes that these findings account for most failures to stain flagella in motile cultures.—*S. H. Hutner.*

HISTOCHEMISTRY

DUNN, R. C. **A hemoglobin stain for histologic use based on the cyanol-hemoglobin reaction.** *Arch. Path.*, **41**, 676. 1946.

The cyanol-peroxidase reaction is specific for hemoglobin, erythrocytes and hemoglobin-containing renal casts and globules. Paraffin sections of tissues fixed in 4% formaldehyde solution buffered to pH 7.0 as well as frozen sections may be used.

The cyanol stock solution is prepared by dissolving 1 g. of cyanol (C.I. No. 715, National Aniline) in 100 ml. of distilled water, adding 10 g. of zinc powder and 2 ml. of C. P. glacial acetic acid. The mixture is boiled until complete decolorization occurs. This solution is stable for several weeks. The working reagent is prepared just before use by filtering 10 ml. of stock solution, adding 2 ml. of glacial acetic acid and 1 ml. of 3% H_2O_2.

Sections are stained as follows: deparaffin and hydrate sections; stain 3–5 min. in freshly prepared working reagent, rinse in water; counterstain 1 minute in 0.1% safranin in 1% acetic acid; rinse in water; dehydrate with alcohols; clear in xylene and mount in clarite.

Hemoglobin is stained dark blue to bluish gray, the nuclei red and the cytoplasm light pink.—*L. Farber.*

McMANUS, J. F. A. **Histological demonstration of mucin after periodic acid.** *Nature*, **158**, 202. 1946.

This note describes the histological demonstration of mucin by Schiff's reagent following the action of periodic acid. Zenker-formol sections were passed to water, after iodine and hypo, and placed for 2 min. in a 0.5% solution of periodic acid in distilled water. The sections were then washed in tap and distilled water and placed in Schiff's reagent for 15 min. at room temperature. The customary rinsings in sulphurous acid, as for the Feulgen test, followed, and the sections were dehydrated in alcohols and mounted in balsam after xylene.

The mucus of the goblet cells of the human intestine and bronchus colored strongly, as did mucous salivary glands, certain pituitary cells, the colloid of the pituitary stalk and thyroid, granules in some nerve cells in the medulla of the rat and in the human intestine, and the basement membranes of the tubular epithelium and of the glomerulus in the kidney.—*J. F. A. McManus.*

SEMMENS, C. S. **Cytology and microchemistry.** *Microscope* **6**, 58–61. 1946.

"Microchemistry" as here used implies the biochemical study of such microscopically demonstrable structures as membranes, chondriosomes, chromosomes and nucleoli. Extracts from such physically dissimilar sources must differ, even if only slightly, in their ultimate chemical constitution. While the direct study of living material will be the ideal ultimate procedure, the many technical difficulties still to be surmounted make fixed material more promising of immediate results. Because of their relative simplicity, Levitsky's fixative and alcohol-acetic mixtures are suggested for preparing material for biochemical study.—*C. E. Allen.*